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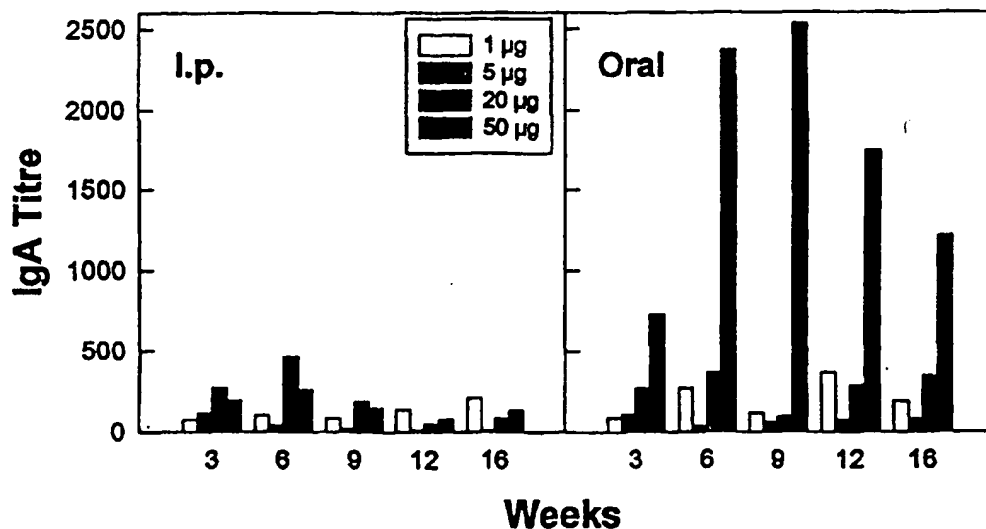
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(71) Applicant (for all designated States except US): MICROBIOLOGICAL RESEARCH AUTHORITY CAMR (CENTRE FOR APPLIED MICROBIOLOGY & RESEARCH) [GB/GB]; Porton Down, Salisbury, Wiltshire SP4 0JG (GB).		Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(72) Inventors; and (75) Inventors/Applicants (for US only): FARRAR, Graham, Henry [GB/GB]; Microbiological Research Authority CAMR (Centre for Applied Microbiology & Research), Porton Down, Salisbury, Wiltshire SP4 0JG (GB). JONES, David, Hugh [GB/GB]; Microbiological Research Authority CAMR (Centre for Applied Microbiology & Research), Porton Down, Salisbury, Wiltshire SP4 0JG (GB). CLEGG, James, Christopher, Stephen [GB/GB]; Microbiological Research Authority CAMR (Centre for Applied Microbiology & Research), Porton Down, Salisbury, Wiltshire SP4 0JG (GB).			

(54) Title: **MICROENCAPSULATED DNA FOR VACCINATION AND GENE THERAPY**



(57) Abstract

A microparticle contains DNA coding for a polypeptide and oral administration of the microparticle leads to its expression. DNA coding for an immunogen is for stimulating antibody formation in a recipient and DNA coding for a non-immunogenic polypeptide is for gene therapy applications. DNA is incorporated into the microparticle without destruction of its function.

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MICROENCAPSULATED DNA FOR VACCINATION AND GENE THERAPY

The present invention relates to microencapsulated DNA, to vaccines comprising microencapsulated DNA, to methods of vaccination and to methods of gene therapy comprising administration of DNA in microparticles, to methods of preparing microparticles containing DNA and to dried compositions comprising DNA-containing particles.

The bio-degradable polymer poly (DL-lactide-co-glycolide) (PLG) has been used for many years by the pharmaceutical industry to deliver drugs and biologicals in microparticulate form *in vivo*. The United States FDA has recently approved a PLG microsphere 30-day delivery system for leuprolide acetate (Lupran Depot (registered trade mark)) to be used in the treatment of prostate cancer. A useful review of the potential of polymer microencapsulation technology for vaccine use is found in Vaccine, 1994, volume 12, number 1, pages 5-11, by William Morris et al.

As an alternative to encapsulation, it is also known to deliver antigens in phospholipid vesicles called liposomes, as described for example by Eppstein, D.A et al in Crit. Rev. Ther. Drug Carrier Syst. 1988, 5(2), pages 99-139. It is reported that a number of antigens have been delivered intraperitoneally using liposomes, including cholera toxin, malaria sporozoite protein and tetanus toxoid, and that influenza antigen has been delivered intra-nasally.

It is also known that, in certain circumstances, injection of naked DNA into tissue can lead to expression of a gene product coded by that DNA. For example, in 1984, work at the United States NIH reported that intrahepatic injection of naked, cloned plasmid DNA for squirrel hepatitis produced both viral infection and the formation of anti-viral antibodies in the squirrels.

WO-A-95/05853 describes methods, compositions and devices for administration of naked polynucleotides which encode biologically active peptides. This published application describes, *inter alia*, the injection of naked DNA coding for an immunogenic

- 2 -

antigen with the aim of raising antibodies in the recipient of the naked DNA.

Liposomal delivery of DNA is also known, and is described, for example, in EP-A-0475178.

An alternative method for obtaining expression of a desired gene product is described in EP-A-0161640, in which mouse cells expressing bovine growth hormone are encapsulated and implanted into a cow to increase milk production therein.

EP-A-0248531 describes encapsulating linear poly (I:C) in microcapsules and using these to induce production of interferon.

WO-A-94/23738 purports to describe a microparticle containing DNA in combination with a conjugate that facilitates and targets cellular uptake of the DNA. In working examples, bombardment of cells by microparticles containing Tungsten is described. These examples appear little different to conventional bombardment of cells with DNA-coated metal particles. Furthermore, sonication is proposed in microparticle manufacture, a step that is known to risk DNA damage but the presented data is inadequate and inappropriate to determine the integrity of the encapsulated DNA.

In the present invention, it is desired to deliver, *in vivo*, DNA encoding proteins with immunogenic, enzymatic or other useful biological activity, usually under the control of an active eukaryotic promoter. Objects of the invention include improvement on vaccination therapies known in the art and improvement upon prior art gene therapy methods.

Improvement of or alternatives to existing compositions and methods are desirable as these existing methods are known to contain a number of drawbacks.

WO-A-95/05853 describes administration of naked polynucleotides which code for desired gene products. However, the compositions and methods in this publication are suitable only for injection, requiring sterile procedures, being in itself an unpleasant

and awkward route of administration.

WO-A-94/23738 purports to provide a process in which encapsulated DNA is released from the particles in the body of the recipient and then taken up by cells, although no accomplished *in vivo* examples are presented.

The invention seeks to provide novel compositions and methods of administration thereof that improve upon existing vaccination and gene therapy techniques and are effective *in vivo*, or at least overcome some of the problems or disadvantages identified in known compositions and methods.

It is known that DNA is readily damaged so that it is no longer capable of inducing expression of a gene product. Surprisingly, the inventors have succeeded in devising a technique for encapsulation of DNA within polymer particles, such that the DNA retains sufficient integrity to induce expression of a gene product coded thereby. The inventors have also succeeded in devising a DNA-containing microparticle suitable for mammalian vaccination or for gene therapy.

Accordingly, a first aspect of the invention provides a pharmaceutical composition comprising DNA encapsulated in a polymer, said DNA comprising a sequence coding for a polypeptide and wherein the composition is adapted to induce expression in a recipient of the coding sequence. Preferably, the coding sequence is accompanied by a promoter promoting expression of the sequence. Where the pharmaceutical composition is for use on mammals, it is convenient to use a eukaryotic promoter and especially a promoter that operates in a wide variety of tissue types. In particular embodiments of the invention, a tissue - or cell type - specific promoter is used.

In use, the pharmaceutical composition is orally administered, and the coding sequence is expressed leading to desired therapeutic effects.

A composition of the invention suitable for vaccination contains a sequence coding for an immunogen. Following administration of the composition, expressed immunogen

elicits production of antibodies within the recipient, thereby contributing to vaccination of the recipient.

A specific embodiment of the invention, described in an example below, contains a DNA sequence coding for a protein. It has been administered *in vivo* and has been found to induce expression in a mammal of that protein. The expression was detected by measurement of production of antibodies specific for that protein. The composition of the specific embodiment comprises microparticles in the size range up to about 10 μm .

Generally, the microparticles of the invention are intended to enter cells of the recipient by phagocytosis, for example phagocytosis by macrophages or other antigen presenting cells. Subsequently, the body of the microparticle breaks down in the intracellular space and the DNA is released. It is preferred that the microparticles of the invention are in the size range 0.01 μm to 30 μm , with 1 μm to 10 μm being a more preferred range. These sizes have been found to be suitable for reliably achieving *in vivo* expression of the DNA. It is also to be noted that agents promoting uptake of the DNA are not needed in microparticles of the invention – as the microparticle size determines its uptake.

An alternative composition, according to the invention, contains a sequence coding for a non-immunogenic product. Such a composition is particularly useful for gene therapy, wherein it is desired to express in a recipient a gene product that is non-expressed, or expressed at a level that it is desired to increase. In this case, a composition of the invention comprises a DNA sequence coding for a desired product, and wherein following administration of the composition expression of the coding sequence results in an increased level of the desired product, giving the gene therapy effect.

It is again a feature of the invention that microparticles for gene therapy applications have sizes in the range 0.01 μm to 30 μm , preferably 1 μm to 10 μm , to target their uptake by phagocytosis.

Specific embodiments of the invention, for use in vaccination, comprise a DNA sequence coding for a protein or an immunogenic component thereof, or an immunogenic fragment or variant thereof, of a virus, bacterium or other pathogenic microorganism. The protein is, for example, an HIV protein, an influenza virus protein, a measles virus protein, a hepatitis virus protein (such as hepatitis surface antigen) or a pertussis protein. Further, where the composition is for oral use, it can conveniently also contain a taste - enhancing agent. The term "taste - enhancing agent" is intended to encompass sweeteners, flavourings and agents that mask any unpleasant taste from other components of the composition. It can conveniently be enterically coated or co-administered with an appropriate antacid formulation.

In a specific embodiment described below, a preparation of microparticles contains a DNA sequence coding for a measles protein. Oral administration of the microparticles elicited an increase in antibodies specific for that protein. Likewise, another microparticle preparation contains a DNA sequence coding for a rotavirus protein. Oral administration of these microparticles preparation elicited anti-rotavirus protein antibodies and a protective effect against challenge by the virus.

Specific embodiments of the invention, suitable for gene therapy, comprise a DNA sequence coding for an enzyme or another protein needed for the treatment of genetic disease. For example, a DNA sequence coding for glucocerebrosidase is suitable for the treatment of Gaucher's disease.

The inventors have thus provided DNA encapsulated within a polymer such that the ability of DNA to code for a desired gene product is substantially not affected by the encapsulation process. It is known that DNA can readily be damaged by emulsifying and other steps necessary for production of polymer particles. The inventors have provided for encapsulation of DNA such that sufficient operative DNA is encapsulated for a biological effect to be obtainable upon administration of the encapsulated DNA.

The invention offers advantages, in that encapsulated DNA is suitable for oral administration, avoiding the unpleasant and awkward aspects associated with having

to inject DNA preparations described in the prior art. Specific embodiments in examples described below have been successful in inducing immunogen-specific antibodies in response to oral administration of a composition of the invention. In addition, the encapsulated DNA formulation is suitable for drying, e.g. freeze drying, into a form that is stable over long periods and is suitable for storage. Further, for many vaccine applications it would be advantageous if, as well as a systemic humoral and cell - mediated immune response, immunity at mucosal surfaces could also be evoked. Specific embodiments of the invention, described below, have been demonstrated to elicit significant increases in specific IgA antibodies, following oral administration. The invention thus provides a pharmaceutical composition comprising DNA within a polymer particle, the DNA encoding a polypeptide, and the composition being adapted to induce mucosal polypeptide specific IgA antibodies in a recipient.

The polymer of the microparticle of the invention preferably is both biodegradable and non-toxic. Suitable polymers include lactide containing polymers and glycolide containing polymers and copolymers of 0-100:100-0 lactide:glycolide. In a specific embodiment of the invention, the polymer comprises poly (DL-lactide-co-glycolide), otherwise referred to as PLG, chosen as it has been approved for human and veterinary use.

The products of the invention are typically for In vivo use on animals, in particular animals. The polymer of the microparticle should therefore be non toxic in vivo and suitable for pharmaceutical use. The polymer should further be biodegradable - either by consisting of or comprising biodegradable polymer - so that it releases its DNA in the recipient. There exists in the art an extensive literature on polymers suitable for human and animal oral use and a person of skill in the art will be able to adapt polymers of the art into the microparticles of the present invention without difficulty. In this connection, the disclosures of EP-A-0451390, WO-A-95/31184 and WO-A-95/31187 are incorporated herein by reference.

The DNA contained within the particle will typically comprise double stranded DNA. The construction of a suitable DNA sequence for use in the invention will be

- 7 -

appreciated by persons of skill in the art. It is preferred that the sequence comprises both a transcriptional promoter and a gene coding sequence. It is further preferred that the DNA sequence provides for a transcription termination and polyadenylation downstream of the coding sequence.

It is particularly preferred that the DNA be double stranded, circular and super coiled. It has been observed that during manufacture of particles the DNA is subjected to severe shear forces. Using particular mild particle manufacturing conditions, the inventors have managed to retain functional DNA, though have observed that previously supercoiled DNA may become partly converted to the open circular form in the process.

Plasmid DNA is particularly suitable and is used in the specific embodiments of the invention described below. As there is extensive literature relating to plasmid manufacture a person of skill in the art will readily be able to prepare a plasmid suitable for the microparticle of the invention. In general, plasmids incorporating any eukaryotic promoter sequence are suitable.

A further optional feature of the invention is that DNA - containing polymer particles can be manufactured so as to have different half-lives *in vivo*. When administering an antigen during vaccination, it may be advantageous for the antigen to be delivered over as long a time frame as possible. A particular embodiment of the invention provides a vaccine comprising first and second vaccine components, the first vaccine component comprising polymer - encapsulated DNA wherein the DNA includes a sequence coding for an immunogen and wherein the polymer has a first half life *in vivo*, and a second vaccine component comprising polymer - encapsulated DNA, wherein the DNA contains a sequence coding for an immunogen and wherein the polymer has a second half - life *in vivo*. The respective half-lives could be up to 5 days and more than 5 days. In one example, the immunogen of the first and second vaccine components are the same. Alternatively, the respective vaccine components can contain DNA sequences coding for different immunogens.

In an embodiment of the invention, the half-lives of the respective first and second vaccine components are up to two days, and more than two weeks. In a further embodiment, the first and second half-lives differ by at least an order of magnitude.

In use of a specific embodiment of the invention, described in an example below, a plasmid encoding luciferase is under control of the human cytomegalovirus immediate early promoter and is encapsulated within PLG in particles around two μm in size. This encapsulated DNA was administered to mice and elicited anti-luciferase antibodies that were detected over a period of several weeks. The production of antibodies in response to encapsulated DNA according to the invention was compared with antibody production in response to administration of naked DNA intraperitoneally and orally. In both cases, encapsulated DNA elicited equivalent or significantly higher amounts of IgG and IgM, and also evoked a significant IgA response.

A second aspect of the invention provides a method of encapsulating DNA in a polymer such that biological activity of the DNA is retained to a significant extent. In an embodiment of the second aspect, a method for encapsulating DNA within a polymer particle, said DNA being capable of inducing expression of a coding sequence within said DNA, comprises preparing a (water-in-oil)-in-water emulsion to form microparticles and separating subsequently produced DNA-containing microparticles by centrifugation. Resultant microparticles preferably have sizes in the range 0.01 μm to 30 μm , more preferably 1 μm to 10 μm .

The method of the invention is carried out under conditions that ensure at least a portion of the DNA is not damaged during manufacture of the particles and thereby retains its ability to induce expression of its gene coding sequence.

It is essential that DNA is incorporated into the microparticles, and DNA incorporation is increased by preparing a solution of DNA plus an alcohol, adding microparticle polymer and forming microparticles therefrom. The alcohol content of the solution suitably varies between 1% and 60% and preferably between 5% and 40%. In specific embodiments of the invention the alcohol content is around 15-35%, more particularly

20-30% for microparticles made from PLG, producing DNA incorporation of 25% and above, up to 50-60%. Ethanol is particularly suitable; methanol and propanol and other alcohols that do not denature DNA are also suitable, and the alcohol is preferably a straight chain or branched C₂-C₁₀ alcohol.

It is also preferred that the emulsification step or steps of the method be carried out under conditions of reduced shear stress, and this is optionally achieved by use of an emulsifying speed that is sufficient to obtain an emulsion and to form microparticles in the desired size range but not so high that all DNA is damaged by excessive shear. In an embodiment of the invention described below the emulsifying mixer speed is modified so that at least 25% DNA activity (assayed by transformation of competent bacteria or transfection of cultured cells) is retained in the resultant microparticles that contain DNA. Suitable speeds are below 8000 rpm, preferably below 6000 rpm, and in a specific embodiment described below the speed is about 3000 rpm.

The method may be performed at ambient temperature, which is convenient for laboratory and industrial purposes, and may also be performed at below ambient temperature improves the stability of the plasmid DNA during the encapsulation procedures. The temperature of the method may be reduced to below 20°C, below 10°C or even below 5°C. In an embodiment of the invention, the method is carried out at below ambient temperature using a reduced amount of microparticle precursor compared to the amount used at ambient temperature.

The parameters of the method are thus chosen to promote formation of microparticles of 10µm diameter or less and to promote incorporation of DNA into microparticles, and to avoid damage to the DNA such that the DNA can not be expressed in the recipient.

For any particular choice of polymer and DNA variations in the method may be necessary to obtain best results. The efficiency of a method can be assessed by transformation or transfection assays. In the transformation assay used by the inventors, DNA is recovered from microparticles by dissolution with organic solvent, quantitated and used to transform bacteria - ampicillin selection determines successful

transformants. In the transfection assay, recovered DNA is used to transfect eukaryotic cells in culture, which culture is then assayed for presence of the antigen or gene therapy product. These assays have demonstrated that DNA recovered from microparticles produced by the method of the invention can retain 50–60% and up to 80% of the activity of the original DNA, indicating high efficiency of incorporation of functional DNA into microparticles.

In a further embodiment of the invention there is provided a method of making a pharmaceutical composition comprising preparing a DNA construct for expression of a coding sequence within the construct, and forming around the construct a polymer particle of size between $0.01\mu\text{m}$ and $30\mu\text{m}$, wherein the construct remains capable of inducing expression of the coding sequence. In use, when the construct is separated from the particle, it induces expression of the coding sequence. The particle is preferably formed by emulsifying a solution of a polymer plus DNA plus alcohol.

The method of the invention is adapted to produce pharmaceutical compositions of the first aspect of the invention. The steps of the method are adapted so that, in a resultant composition which contains many DNA containing polymer particles, a useful proportion of particles contain active DNA, i.e. DNA that has not been damaged by the method such that its ability to induce expression of its coding sequence is lost. DNA activity is measured as a percentage of activity prior to the particle forming step.

An acceptable level of DNA biological activity is at least 10% and preferably at least 25%, though for particularly fragile DNA a lower percentage may be acceptable so long as, in use, a therapeutic effect is demonstrated by the composition.

In a specific embodiment of the invention, a composition is made by preparing a solution of a plasmid of double stranded, supercoiled DNA comprising a coding sequence and a eukaryotic promoter. Separately, a polymer solution is prepared. The two solutions are mixed together and emulsified at a speed between 1000 and 4000 rpm. A solution of a stabilizing agent is then added and the new mixture emulsified at a speed between 1000 and 4000 rpm. After centrifugation and resuspension of

particles the DNA within retains 25% of its activity.

A third aspect of the invention provides a pharmaceutical composition comprising polymer - encapsulated DNA and having a reduced water content, such as less than 5% by weight. This composition is suitable for long term storage while retaining the ability of the DNA, upon administration to a recipient, to induce expression of a coding sequence within said DNA.

A method of preparing a pharmaceutical composition for storage, is to dry, such as by freeze drying, a pharmaceutical composition according to the first aspect of the invention. It is preferred that the dried composition has a water content of less than 5%, though the precise water content will be determined by the period of drying used.

A fourth aspect of the invention provides a method of vaccination comprising administering a vaccine according to the first aspect of the invention. Vaccination can thus be obtained by eliciting antibodies to the immunogen expressed from the gene coding sequence. As will be appreciated, the immunogen can be a component of a virus or bacterium or other pathogenic microorganism, or can be an analogue of said immunogen such that antibodies against the analogue are effective against the pathogen itself.

In a fifth aspect of the invention there is provided a method of gene therapy, comprising identifying a gene product that is to be expressed in an individual, preparing a composition according to the first aspect of the invention for expression of the desired gene product and administering said composition. The gene product desired to be expressed is typically a gene product that is previously not fully or not adequately expressed, or not expressed at all, in the patient receiving the gene therapy.

The gene therapy can conveniently be achieved by administration orally or intranasally, or by injection.

According to a sixth aspect of the invention there is provided use of a microparticle according to the first aspect of the invention in manufacture of a medicament for inducing production of IgA antibodies.

According to a seventh aspect of the invention there is provided use of a microparticle according to the first aspect of the invention in manufacture of a medicament for gene therapy.

In a specific embodiment of the invention described in an example below, the particle material is PLG. The size of particles produced by the method of the invention are generally in the range of 0.01–30 μm , preferably 1–10 μm . Other suitable polymer formulations for DNA – containing particles according to the present invention include poly-lactic acid, poly – hydroxybutyrate, poly hydroxyvalerate, poly (hydroxybutyrate/valerate), ethyl cellulose, dextran, polysaccharides, polyalkylcyanoacrylate, poly-methyl-methacrylate, poly(ϵ -caprolactone) and mixtures of all of these components.

In use of a specific embodiment of the invention, described in an example below, a preparation of microparticles according to the invention comprises DNA coding for the protein luciferase. As will be appreciated by a person of skill in the art, a wide range of DNA sequences and constructs are suitable for use in this invention. In particular, the invention can be practised incorporating a wide range of plasmid vectors already well known and characterised in the art. Typically, a plasmid vector used in this invention will include a cDNA that codes for the desired gene product. The selection of additional components for the DNA sequence, such as promoters, reporter genes and transcription termination sequences can be made by a person of skill in the art according to common general knowledge concerning construction of known plasmid vectors.

The preferred administration route for compositions of the invention is the oral route, meaning that compositions of the invention should preferably be designed to avoid significant degradation while passing through the stomach with its high acid levels.

It is known that uptake of microparticles of less than 10 μm in size occurs, *inter alia*, in the M cells of the intestine, and thus inclusion of DNA containing particles in this size range can be advantageous in promoting uptake at this intestinal location. Other modifications to the nature and character and components of the polymer can be made within the concept of the invention.

There now follows description of specific embodiments of the invention, accompanied by figures in which:-

Fig.1 is a schematic diagram of the components of a protein - expressing plasmid suitable for incorporation into a DNA-containing particle according to the invention;

Fig. 2 illustrates the results of example 3, namely induction of luciferase - specific serum antibodies by PLG - encapsulated plasmid DNA, and in which the left column indicates antibody titre after 3 weeks and the right column indicates antibody titre after 6 weeks (i.m. represents intra muscular, i.p. represents intra peritoneal, the bottom portion of each column represents IgG levels, the top portion represents IgM levels);

Fig. 3 shows dose-response data for injected and oral doses of encapsulated DNA, with the titre of IgG, IgM and IgA in each case.

Fig. 4 shows: A - agarose gel electrophoresis of plasmid DNA following homogenisation in a Silverson mixer at 2000 and 8000 rpm for 0 - 300 seconds; and B - agarose gel electrophoresis of DNA before and after encapsulation;

Fig. 5 shows stool anti-luciferase IgA response to DNA within PLG microparticles.

Fig. 6 shows the results of oral administration of PLG - encapsulated DNA

expressing measles virus N protein; and

Fig. 7 shows the results of oral administration of PLG - encapsulated DNA expressing rotavirus VP6 gene: A - faecal rotavirus - specific IgA response, B - rotavirus shedding after challenge of orally immunized mice.

Example 1

Method for Encapsulation of Plasmid DNA In PLG microparticles

Equipment:

- 1) Silverson Laboratory mixer with 3/4" probe fitted with emulsor screen.
- 2) High speed centrifuge.
- 3) Normal laboratory glassware, beakers, measuring cylinders, stirrers etc.

Reagents:

- 1) Poly(lactide-co-glycolide) (PLG) solution - 500 mgs in 3 ml dichloromethane.
- 2) Plasmid DNA (12 mg/ml in water).
- 3) Polyvinyl alcohol (PVA) solution (8% w/v in water).
- 4) Absolute ethanol.
- 5) TEN buffer (10 mM tris pH 8.0 + 1 mM EDTA + 50 mM NaCl).

Method:

- 1) Mix 200 μ l plasmid DNA solution with 250 μ l of TEN and add 150 μ l ethanol with stirring. Mix well.
- 2) Add this mixture to 3 ml PLG solution and emulsify in the Silverson mixer at 3000 rpm for 2 min.
- 3) Add this emulsion to 100 ml PVA and emulsify at 3000 rpm for 2 min.
- 4) Add the double emulsion to 1 litre of water and stir vigorously for 1 min.
- 5) Distribute the suspension of microparticles in centrifuge containers and centrifuge at 10,000 x g_{av} for 30 mins.
- 6) Resuspend the microparticle pellet in 25ml of water and homogenise with a hand homogeniser with large clearance (0.5mm) to make a homogeneous suspension.

- 15 -

Dilute with 200 ml of water and recentrifuge as above.

7) Repeat step 6 three times.

8) Resuspend the microparticle pellet in 25 ml of water as above, transfer to a vessel suitable for freeze drying, shell freeze in an isopropanol/dry ice mixture and lyophilise for 48 h.

In this method, steps 1-3 are carried out at ambient temperature. DNA was incorporated into the microparticles with an efficiency of about 25%.

Example 2

Plasmids for the Expression of Proteins after *In Vivo* Delivery

Suitable plasmids for use in microparticles according to the invention consist of the following components (see fig. 1):-

1. Plasmid Backbone The plasmid backbone has an origin of replication and an antibiotic resistance gene or other selectable marker to allow maintenance of the plasmid in its bacterial host. Backbones providing a high copy number will facilitate production of plasmid DNA. An example would be from the pUC plasmid vectors.

2. Transcriptional Promoter Sequence Expression of the desired protein will be driven by a, typically eukaryotic, transcriptional promoter initiating the synthesis of mRNA. Generally, strong promoters functioning in a wide variety of tissue types and animal species are to be used, e.g. the human cytomegalovirus immediate early (hCMV IE) promoter. However, particularly for gene therapy applications, a tissue- or cell type-specific promoter may be more appropriate.

3. Coding Sequence The coding sequence contains the DNA sequence encoding the protein of interest. It contains the translational start codon ATG in sequence context favourable for initiation of protein synthesis. The coding sequence ends with a translational termination codon. Proteins to be expressed include a) reporter enzymes (e.g. luciferase, β -galactosidase); b) components of pathogenic microorganisms

capable of inducing protective immune responses (e.g. the NS1 protein of tick-borne encephalitis virus, the N, H or F proteins of measles virus, the gp120 protein of human immunodeficiency virus 1); c) enzymes or other proteins intended for the treatment of genetic disease (e.g. glucocerebrosidase for the treatment of Gaucher's disease).

4. Transcription Termination Sequence Improved expression levels are obtained under some circumstances when sequences causing termination of mRNA transcription are incorporated downstream of the coding sequence. These sequences frequently also contain signals causing the addition of a poly A tail to the mRNA transcript. Sequences which can be used in this role can be derived from the hCMV major immediate early protein gene or from SV40 viral DNA or elsewhere.

Example 3

We have constructed a plasmid encoding the insect protein luciferase, under the transcriptional control of the human cytomegalovirus immediate early (hCMV IE) promoter, and demonstrated luciferase activity in cells transfected *in vitro*.

We encapsulated purified plasmid DNA in PLG microparticles around 2 μ m in size with moderate (about 25%) efficiency using the protocol of Example 1. Agarose gel electrophoresis indicates that a proportion of the initially closed circular supercoiled DNA undergoes conversion to a more slowly migrating form, probably relaxed circles, as a result of shear stresses in the encapsulation process. The encapsulated DNA was released from the particles and shown to retain a significant fraction of its *in vivo* biological activity in assays of bacterial transformation by electroporation, and luciferase expression after transfection into cultured cells.

Microencapsulated DNA (50 μ g) was administered to mice by intraperitoneal (i.p.) injection and orally. Control animals received unencapsulated DNA by the same routes, and as a positive control by standard intramuscular (i.m.) injection. Luciferase-specific serum antibodies were analyzed by ELISA three and six weeks after DNA administration. Results are presented in fig. 2.

- 17 -

As shown in fig. 2, modest specific IgG and IgM responses were seen after i.m. injection, as might be expected. Encapsulated DNA evoked strong IgG and IgM responses after i.p. injection, while unencapsulated DNA gave much weaker responses. Similarly, orally administered encapsulated DNA evoked a good IgG response which was not matched by unencapsulated DNA. The IgG and IgM antibody responses indicate that luciferase expression and presentation to the immune system occur after administration of plasmid DNA encapsulated in PLG microparticles with efficiencies exceeding that seen with the standard i.m. route, and exceeding that seen in comparative administration of unencapsulated DNA.

Example 4

In further experiments, microencapsulated DNA, made by the method of Example 1, in a range of doses (1–50 μ g DNA) was administered to groups of outbred mice by intra-peritoneal (i.p.) injection or orally. Luciferase-specific serum antibodies of IgG, IgM and IgA classes were analysed by ELISA at 3,6 and 9 weeks after DNA administration.

In Fig. 3, it can be seen that i.p. injection of PLG-encapsulated DNA evoked good IgG and IgM responses, and a modest IgA response. Orally administered encapsulated DNA evoked good responses in all three antibody classes. There is a trend for the antibody titres to increase with time after DNA administration, and the responses are also dose-related to a greater or lesser extent. It is apparent that quantities of DNA as low as 1 μ g are able to evoke significant responses, especially at longer times after administration. These antibody responses again confirm that luciferase expression occurs after administration of plasmid DNA encapsulated in PLG microparticles, either by i.p. injection or orally. They also demonstrate that antigen is presented to the immune system by these means in such a fashion as to evoke IgG, IgM and IgA classes of antibody.

Example 5

We examined the effect of high-speed homogenisation steps, used to generate the required water-oil-water emulsions which are intermediates in the encapsulation process, on the physical integrity and biological function of plasmid DNA.

In initial experiments, supercoiled plasmid DNA was adjusted to concentrations and volumes similar to those to be used in microencapsulation experiments, and homogenised with a Silverson laboratory homogeniser. Samples were removed at intervals from 0 to 300 sec for analysis by agarose gel electrophoresis (Fig. 4A). Such an analytical procedure is capable of distinguishing between supercoiled (sc) DNA, open circular (oc) DNA, where a single strand has been nicked, and linear (l) DNA, where both strands have been cut at adjacent points (see, for example, Fig. 2C in Garner and Chrambach 1992. Resolution of circular, nicked and linear DNA, 4.4 kb in length, by electrophoresis in polyacrylamide solutions. *Electrophoresis* 13, 176-178). It is clear that exposure to such conditions for periods as short as 10 sec results in conversion from sc to oc form. At 8000 rpm, further conversion to the linear form and eventually more extensive degradation occur. However, at the reduced speed of 2000 rpm the oc form of DNA is relatively stable over the time period typically required for formation of the emulsion intermediates involved in PLG encapsulation. These studies thus show that plasmid DNA is vulnerable to shear-induced damage, and careful attention is required to the precise conditions to obtain encapsulation of minimally altered DNA.

From this basis, we have developed conditions for the encapsulation of purified plasmid DNA in PLG microparticles around 2 μ m in size with moderate (about 25%) efficiency. Agarose gel electrophoresis (Fig. 4B) indicates that the initially closed circular supercoiled DNA undergoes conversion to the oc form, as a result of shear stresses in the encapsulation process. Biological activity of DNA released from microparticles has been assessed in assays of bacterial transformation by electroporation, and luciferase expression after transfection into cultured cells. DNA released from the particles retains a significant fraction (about 25%) of its *in vitro*

activity in both these assays.

Example 6

PLG-encapsulated DNA coding for luciferase, made by the method of Example 3, is also able to evoke a mucosal immune response to the expressed protein. Levels of IgG, IgM and IgA antibodies specific for luciferase were assessed by ELISA in stool samples from mice which received i.p. or oral doses of 1, 5, 20 or 50 μ g of PLG-encapsulated DNA. No significant levels of IgG or IgM antibodies were found in stool samples from any group of mice. Rather limited IgA responses were seen in the i.p.-injected mice; however, oral administration resulted in significant levels of luciferase-specific IgA antibodies in the stool samples (Fig. 5). These reached extraordinarily high levels in those mice which received 50 μ g PLG-encapsulated DNA. These results indicate that oral administration of a single dose of PLG-encapsulated plasmid DNA is capable of evoking a mucosal, as well as a systemic antibody response. This may be a useful attribute of a PLG-encapsulated DNA vaccine in applications where protection against infection at mucosal surfaces is desirable, as for measles or AIDS.

Example 7

We have exploited a plasmid expressing the measles virus (MV) nucleocapsid protein (N) to extend our observations that the oral administration of encapsulated plasmid DNA expressing luciferase is capable of eliciting a systemic antibody response. The N-expressing construct is identical to that expressing luciferase (described in example 3), except for the replacement of the coding sequence with the Edmonston strain MV N coding sequence. The purified plasmid DNA was PLG-encapsulated (using the method as described in example 1).

Inbred C3H mice were immunized with two doses suspended in 0.1 M Sodium bicarbonate administered by oral gavage, 13 days apart; each dose contained 50 μ g DNA. Control groups of mice received PBS alone or PLG particles containing plasmid vector DNA containing no coding sequence. Mice were bled at intervals and serum

levels of IgG specific for MV N determined by ELISA, using recombinant MV N expressed in insect cells as antigen. As shown in Fig. 6A, immunization with PLG-encapsulated DNA expressing MV N resulted in significant levels of N-specific antibody; results shown are mean absorbances in 1/100 diluted sera taken 53 days after the second DNA administration. There seems to be a considerable degree of variability in the response of individual mice to DNA immunization in these experiments (see Fig. 6B), but very high levels of antibody (reciprocal titres exceeding 10^4 , determined in follow-up experiments) are present in some animals. These results demonstrate that oral delivery of PLG-encapsulated DNA is an effective method for inducing an immune response against an important pathogen.

Example 8

A Further Method for Encapsulation of Plasmid DNA in PLG microparticles

Equipment:

- 1) Silverson Laboratory mixer with 3/4" probe fitted with emulsor screen.
- 2) High speed centrifuge.
- 3) Normal laboratory glassware, beakers, measuring cylinders, stirrers etc.

Reagents:

- 1) Poly(lactide-co-glycolide) (PLG) solution - 500 mgs in 3 ml dichloromethane.
- 2) Plasmid DNA (>10 mg/ml in TE buffer).
- 3) Polyvinyl alcohol (PVA) solution (8% w/v in water).
- 4) Absolute ethanol.
- 5) TE buffer (10 mM tris pH 8.0 + 1 mM EDTA + 50 mM NaCl).

Method:

- 1) Mix 450 μ l plasmid DNA solution with 150 μ l ethanol with stirring. Mix well.
- 2) Add this mixture to 3 ml PLG solution and emulsify in the Silverson mixer at 2000 rpm for 2½ min.
- 3) Add this emulsion to 100 ml PVA and emulsify at 2000 rpm for 2½ min.
- 4) Add the double emulsion to 1 litre of water and stir vigorously for 1 min.

- 21 -

- 5) Distribute the suspension of microparticles in centrifuge containers and centrifuge at $10,000 \times g_{av}$ for 30 mins.
- 6) Resuspend the microparticle pellet in 25ml of water and homogenise with a hand homogeniser with large clearance (0.5mm) to make a homogeneous suspension. Dilute with 200 ml of water and recentrifuge as above.
- 7) Repeat steps 5 and 6 four times.
- 8) Resuspend the microparticle pellet in 25 ml of water as above, transfer to a vessel suitable for freeze drying, shell freeze in an isopropanol/dry ice mixture and lyophilise for 48 h.

In this method, steps 1–3 are carried out at ambient temperature. The efficiency was improved compared to example 1, up to 30–40% efficiency.

Example 9

A Further Method for Encapsulation of Plasmid DNA in PLG microparticles

Equipment:

- 1) Silverson Laboratory mixer with 3/4" probe fitted with emulsor screen.
- 2) High speed centrifuge.
- 3) Normal laboratory glassware, beakers, measuring cylinders, stirrers etc.

Reagents:

- 1) Poly(lactide-co-glycolide) (PLG) solution – 400 mgs in 3 ml dichloromethane.
- 2) Plasmid DNA (>10 mg/ml in TE buffer).
- 3) Polyvinyl alcohol (PVA) solution (8% w/v in water).
- 4) Absolute ethanol.
- 5) TE buffer (10 mM tris pH 8.0 + 1 mM EDTA).

Method:

- 1) Mix 450 μ l plasmid DNA solution with 150 μ l ethanol with stirring. Mix well.
- 2) Add this mixture to 3 ml PLG solution and emulsify in the Silverson mixer at 2000 rpm for 2½ min.

- 22 -

- 3) Add this emulsion to 100 ml PVA and emulsify at 2000 rpm for 2½ min.
- 4) Add the double emulsion to 1 litre of water and stir vigorously for 1 min.
- 5) Distribute the suspension of microparticles in centrifuge containers and centrifuge at $10,000 \times g_{av}$ for 30 mins.
- 6) Resuspend the microparticle pellet in 25ml of water and homogenise with a hand homogeniser with large clearance (0.5mm) to make a homogeneous suspension. Dilute with 200 ml of water and recentrifuge as above.
- 7) Repeat steps 5 and 6 four times.
- 8) Resuspend the microparticle pellet in 25 ml of water as above, transfer to a vessel suitable for freeze drying, shell freeze and lyophilise for 48 h.

In this method, steps 1–3 are carried out at 4°C. The efficiency of incorporation of DNA into microparticles was 50–60%.

Example 10

Plasmid DNA (pCMVIA/VP6) expressing the VP6 gene of murine rotavirus (epizootic diarrhoea of infant mice (EDIM) virus) was constructed at the University of Massachusetts Medical Center. The gene encoding VP6 was inserted into a vector (pJW4303) downstream of sequences of the immediate early transcriptional promoter and intron A of human cytomegalovirus and a tPA-derived secretory signal sequence. The gene was followed by transcriptional termination and polyadenylation sequences derived from the bovine growth hormone gene. The purified plasmid DNA was PLG-encapsulated using the method described in example 1.

Inbred Balb/c mice were immunized by oral administration with a dose containing 50 micrograms VP6-expressing DNA encapsulated in PLG microparticles. A control group of mice received a similar dose of encapsulated vector DNA. Mice were examined for intestinal rotavirus-specific IgA at fortnightly intervals by ELISA, using EDIM virus as antigen. Nine weeks after immunisation, the animals were challenged with EDIM virus, and monitored for excretion of virus in stool using a monoclonal antibody-based ELISA.

As shown in Fig. 7A, immunisation with PLG-encapsulated DNA expressing VP6 resulted in significant levels of rotavirus-specific intestinal IgA antibody when compared with the control animals. This is particularly noteworthy, since other routes of administration of VP6-expressing plasmid DNA do not elicit detectable levels of intestinal virus-specific IgA before virus challenge. After challenge with EDIM virus, there was a reduction in rotavirus shedding in mice which had received PLG - encapsulated VP6-expressing plasmid DNA, compared with the control group (Fig. 7B).

These results demonstrate that orally administered PLG-encapsulated plasmid DNA is capable of inducing a) a specific intestinal IgA response, and b) protection against virus challenge, as manifested in a reduction in virus shedding.

The compositions and methods of the invention have application in slow release systems for delivery of DNA vaccines; prolonged expression of immunogen potentially results in efficient single dose priming and boosting, with consequent efficient induction of long term memory responses. Another application is in a vehicle for the oral delivery of vaccines; simple and acceptable means for vaccine administration are likely to improve vaccine uptake rates; in addition, freeze-dried encapsulated plasmid DNA is likely to be very stable and insensitive to environmental conditions. A further application is in a slow release system for gene therapy; prolonged release of DNA and subsequent expression potentially reduces the need for repeated treatment.

CLAIMS

1. A composition comprising a microparticle and DNA, wherein the DNA is inside the microparticle and comprises a sequence coding for a polypeptide, and wherein the microparticle is adapted to induce expression of the coding sequence following administration to a recipient.
2. A composition according to Claim 1 adapted to induce expression of the coding sequence following oral administration.
3. A composition according to Claim 1 or 2 comprising circular DNA.
4. A composition according to Claim 3 comprising double-stranded DNA selected from (i) plasmid DNA and (ii) DNA derived from plasmid DNA by one or more of insertion, deletion and substitution.
5. A composition according to any previous claim wherein the DNA comprises a sequence promoting transcription of the coding sequence.
6. A composition according to any previous claim wherein the microparticle is non-toxic and pharmaceutically acceptable and consists of or comprises a bio-degradable polymer.
7. A composition according to Claim 6 wherein the polymer is a lactide containing polymer.
8. A composition according to Claim 6 or 7 wherein the polymer is a glycolide-containing polymer.
9. A composition according to Claim 7 or 8 wherein the polymer comprises poly (DL-lactide-co-glycolide).

- 25 -

10. A composition comprising microparticles according to any of Claims 1-9 wherein at least 50% of the microparticles are in the size range 0.01 μm to 30 μm .
11. A composition according to Claim 10 wherein at least 50% of the microparticles are in the size range 1 μm to 10 μm .
12. A composition according to claim 10 or 11 wherein substantially all microparticles are in the size range less than 30 μm .
13. A composition according to any of Claims 1-12, comprising a DNA sequence coding for an immunogen and adapted to induce expression of that immunogen in a recipient following oral or parenteral administration.
14. A composition according to Claim 13, adapted to induce production of antibodies specific to that immunogen in the recipient.
15. A composition according to Claim 14 adapted to induce production of IgA antibodies.
16. A composition according to any of Claims 13-15 adapted for vaccinating a mammal, wherein vaccination is obtained by production of antibodies by the mammal in response to the immunogen, itself produced by expression of the DNA coding sequence.
17. A vaccine for eliciting antibodies against an immunogen, comprising a composition according to any of Claims 1-16 and a pharmaceutically acceptable carrier, wherein the DNA sequence codes for said immunogen.
18. A vaccine according to Claim 17 wherein said immunogen is an immunogenic component of a virus or a bacterium or another pathogenic microorganism or is an immunogenic fragment or variant thereof.

- 26 -

19. A vaccine according to Claim 18 wherein the DNA sequence codes for a viral protein.
20. A vaccine according to any of Claims 17-19 further comprising a taste-enhancing agent.
21. A vaccine according to any of Claims 17-20, comprising first and second vaccine components, the first vaccine component comprising DNA inside a microparticle wherein the DNA includes a sequence coding for an immunogen and wherein the microparticle has a first half-life *in vivo*, and a second vaccine component comprising DNA inside a microparticle, wherein the DNA contains a sequence coding for an immunogen and wherein the microparticle has a second half-life *in vivo*.
22. A vaccine according to Claim 21 wherein the immunogen of the first vaccine component and the immunogen of the second vaccine component are the same.
23. A vaccine according to Claim 21 or 22 wherein the first and second half-lives are, respectively, up to 2 weeks and more than 2 weeks.
24. A vaccine according to Claims 21 or 22 wherein the ratio of the first and second half-lives is at least 2:1.
25. A composition according to any of Claims 1-12 adapted to induce, in a recipient, expression of a desired, non-immunogenic gene product.
26. A composition according to Claim 25 for inducing expression of a desired non-immunogenic product, which product is (i) previously substantially not expressed, or (ii) previously expressed at a level which it is desired to increase.
27. A composition according to Claim 25 or 26 for treatment or prevention of a disease caused by non-expression or reduced expression or absence of a gene product, wherein the DNA coding sequence of the composition codes for said gene

product.

28. A composition according to any of Claims 25-27 for gene therapy.
29. A composition comprising polymer-encapsulated DNA and having a water content of less than 5% by weight.
30. A composition comprising polymer-encapsulated DNA and having a water content of less than 5%, obtained by freeze-drying a composition according to any of Claims 1-28.
31. A method of preparing a composition for storage, comprising preparing an aqueous solution of polymer particles, said particles containing DNA, and drying the solution to a water content of less than 5%.
32. A method of making a microparticle comprising the steps of:-
- i. preparing a mixture of DNA and microparticle precursor;
 - ii. forming a microparticle that contains DNA; and
 - iii. separating DNA-containing microparticles from the mixture, wherein DNA in the DNA-containing microparticle comprises a sequence coding for a polypeptide and wherein the microparticle is adapted to induce expression of the coding sequence in a recipient.
33. A method according to Claim 32 comprising:-
- a. forming a plurality of microparticles under conditions such that the ability of the DNA to induce expression of its coding sequence is at least 25% of its ability to induce expression of its coding sequence prior to microparticle formation.

- 28 -

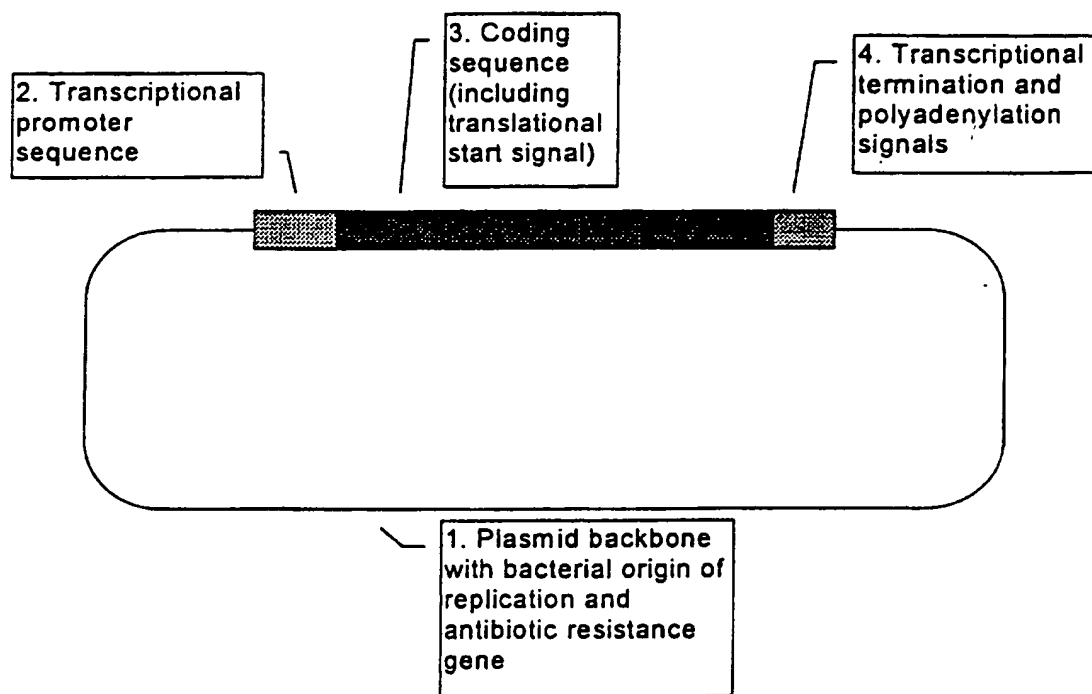
34. A method according to Claim 33 comprising forming the emulsion under conditions of reduced shear stress such that at least 10% of encapsulated DNA retains its ability to induce expression of its coding sequence.
35. A method according to Claim 34 wherein at least 25% of encapsulated DNA retains its ability to induce expression of its coding sequence.
36. A method according to any of Claims 32-35 comprising preparing a mixture of DNA and alcohol, and adding microparticle precursor to the mixture.
37. A method according to claim 36 comprising preparing a solution of DNA and alcohol with an alcohol content of 10-40%.
38. A method according to any of Claims 32-37 comprising forming microparticles in the size range 0.01 μm to 30 μm .
39. A method according to any of Claims 32-38 wherein the DNA is circular, plasmid DNA.
40. A method according to any of Claims 32-39 further comprising freeze drying the microparticle.
41. A method of encapsulating DNA in a microparticle, wherein the DNA comprises a sequence coding for a polypeptide and is adapted to induce expression of the coding sequence, comprising the steps of preparing a mixture of the DNA and a water-in-oil-in-water emulsion suitable to form microparticles, forming microparticles that contain DNA and separating DNA-containing microparticles from the mixture by centrifugation, characterised in that the DNA retains its ability to induce expression of its coding sequence.
42. A method of vaccination, comprising administering a vaccine according to any of Claims 17-24.

- 29 -

43. A method of vaccination according to Claim 42 wherein vaccination is obtained by eliciting antibodies to the immunogen expressed from the coding sequence.
44. A method of gene therapy comprising identifying a gene product that is to be expressed, preparing a composition according to any of Claims 26-28 wherein the coding sequence codes for the gene product and administering the composition.
45. A method according to any of Claims 42-44 wherein administration is oral or intra-nasal.
46. A method according to any of Claims 42-44 wherein administration is by injection.
47. Use of a composition according to any of Claims 1-16 in manufacture of a medicament for inducing mucosal immunity.
48. Use of a composition according to any of Claims 1-16 in manufacture of a medicament for inducing production of IgA antibodies.
49. Use of a composition according to any of Claims 1-6 in manufacture of a medicament for inducing prophylactic or therapeutic immune responses.

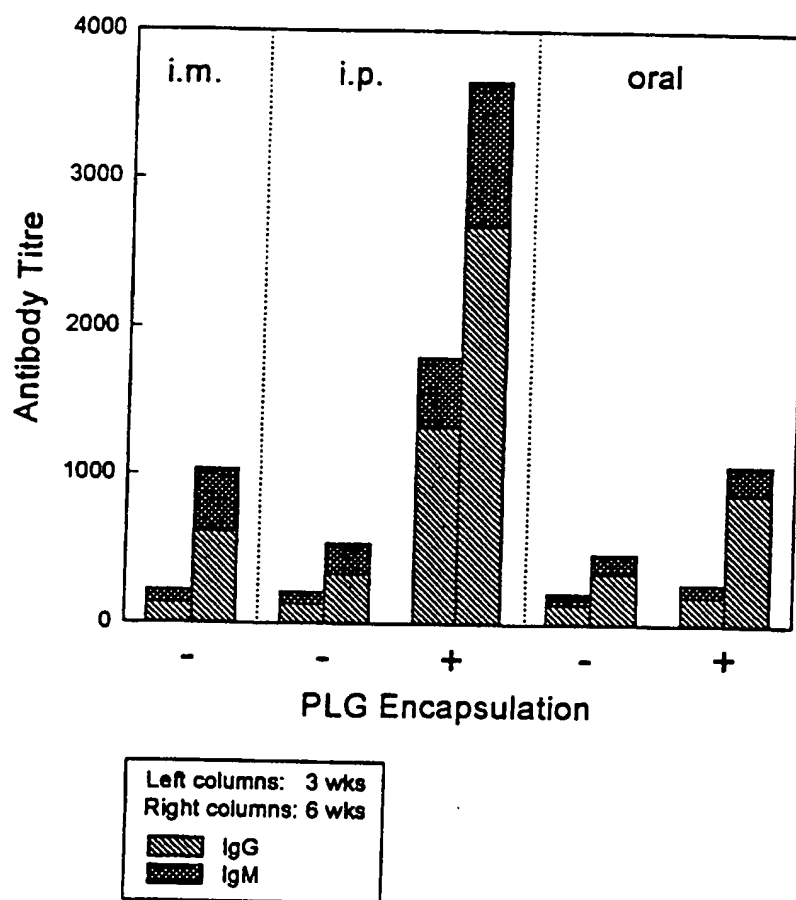
1 / 7

FIG. 1



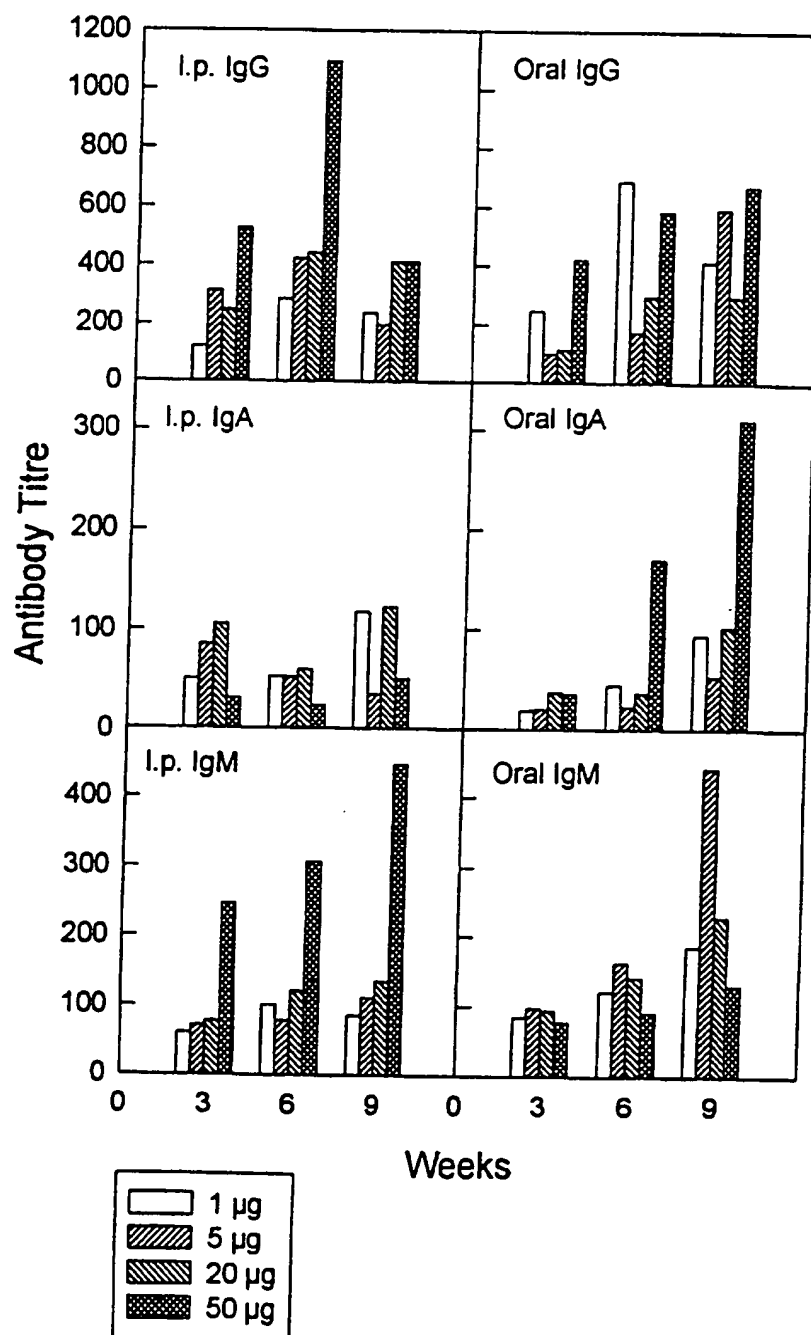
2/7

FIG. 2



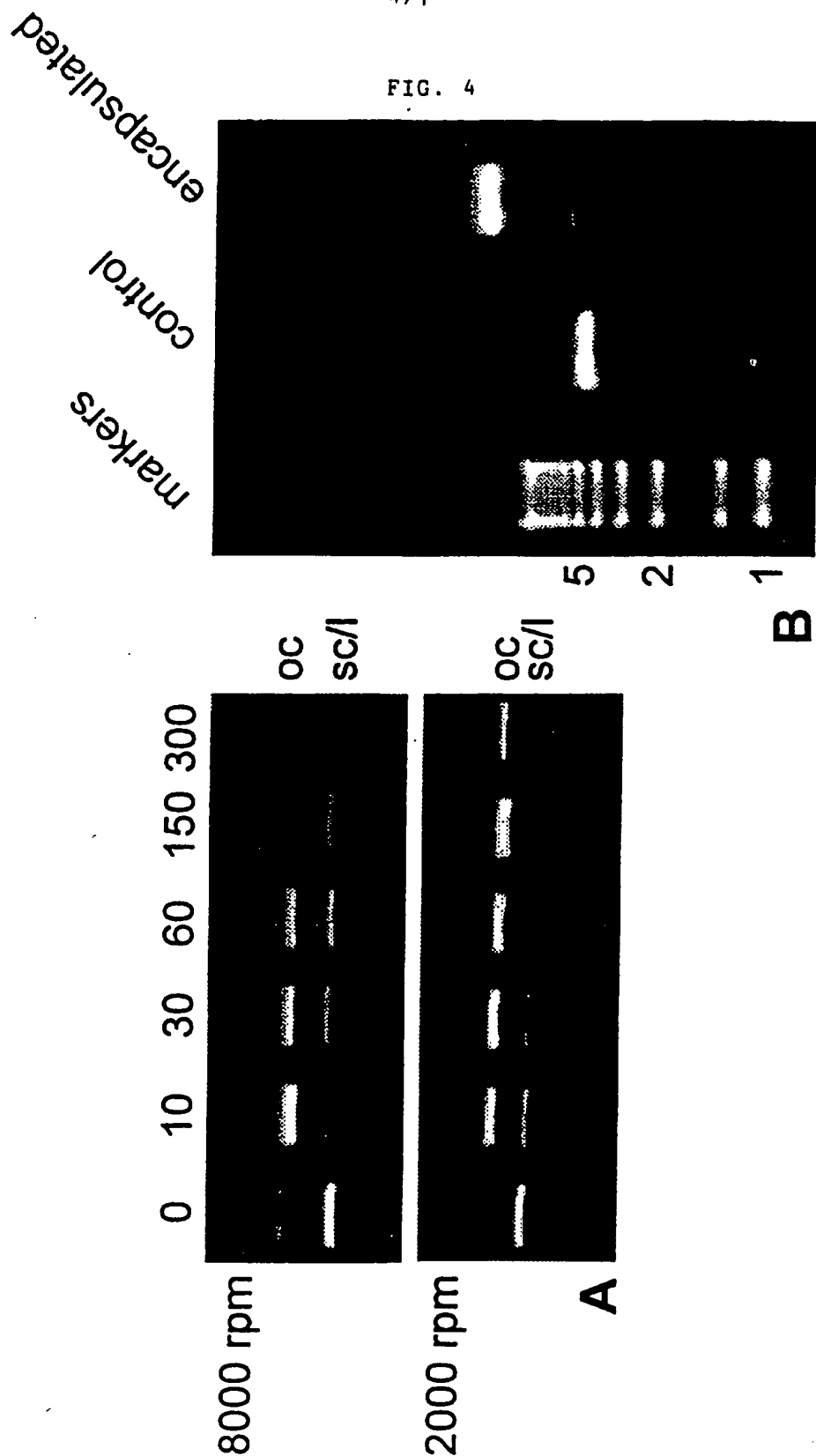
3/7

FIG. 3



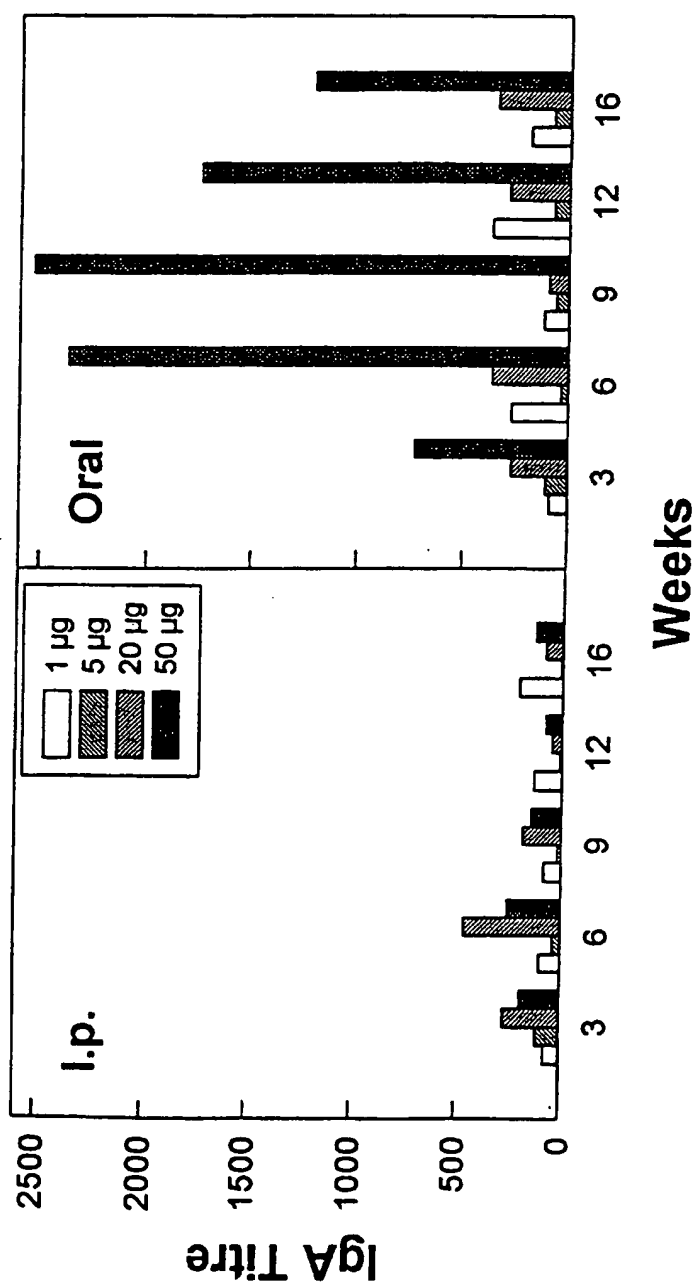
4/7

FIG. 4



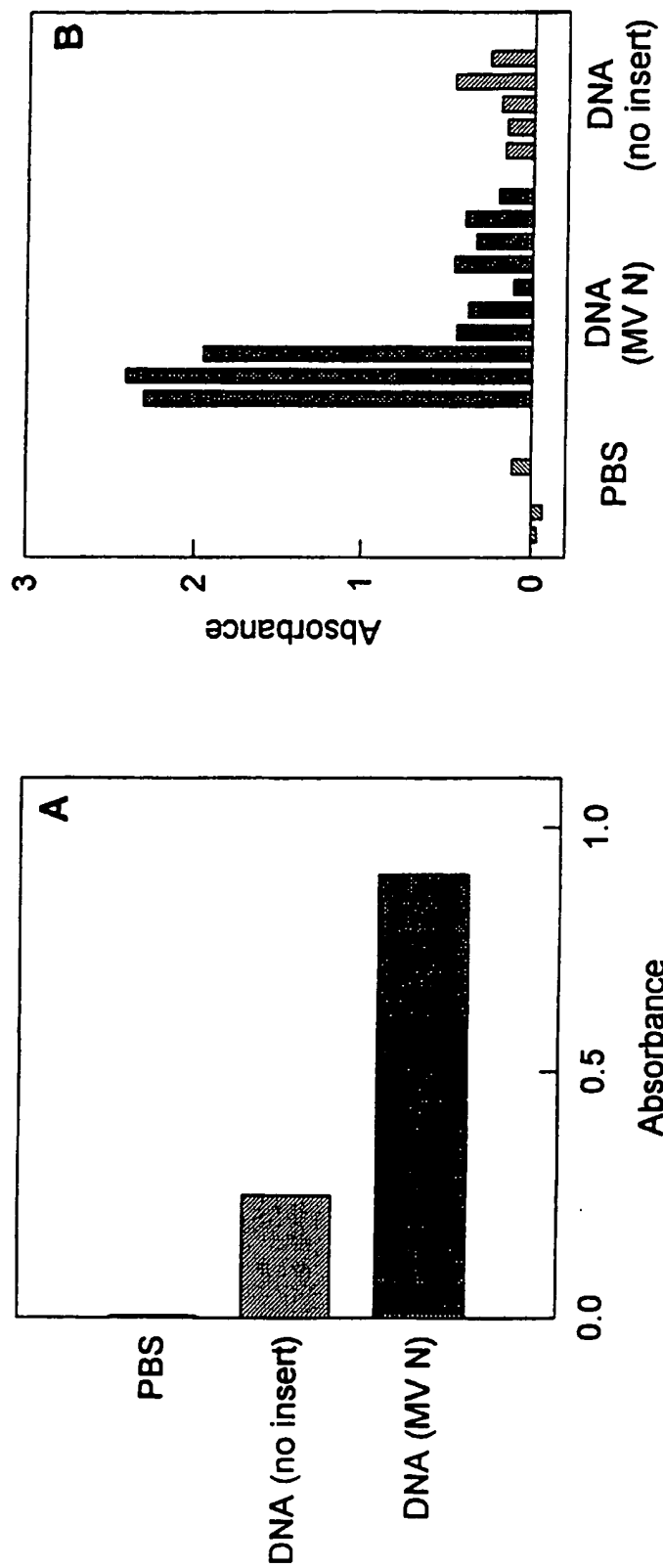
5/7

FIG. 5



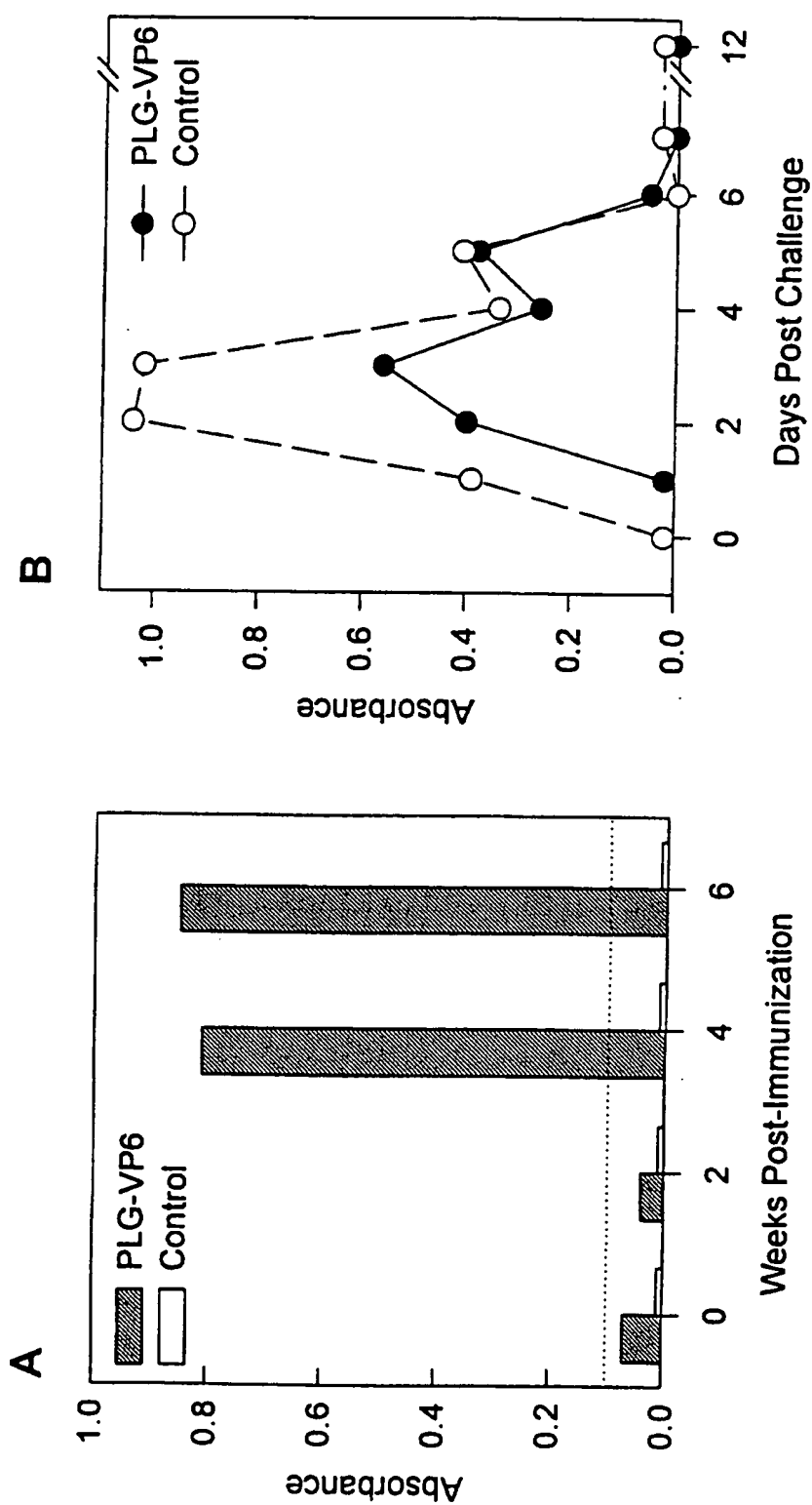
6/7

FIG. 6



7/7

FIG. 7



INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 96/02770

A. CLASSIFICATION OF SUBJECT MATTER
 IPC 6 A61K9/16 A61K9/00 A61K39/15 A61K39/165 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 24929 A (UNIV BROWN RES FOUND) 21 September 1995 see page 5, line 15 - page 19, line 29 ---	1-12, 25-41, 44-46
X	WO 95 20660 A (UNIV MASSACHUSETTS MEDICAL ;ST JUDE CHILDREN S RESEARCH HO (US)) 3 August 1995 see page 1, line 27 - page 3, line 13 see page 6, line 16 - page 13, line 33 see page 40 - page 42; example 9 ---	1-6, 10-24, 42,43, 45-49
X	WO 94 23738 A (MEDISORB TECHNOLOGIES INTERNAT) 27 October 1994 cited in the application see page 4, line 9 - page 7, line 12 see page 8, line 31 - page 30, line 10 ---	1-49
	-/--	



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
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- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"A" document member of the same patent family

Date of the actual completion of the international search

28 February 1997

Date of mailing of the international search report

11. 03. 97

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
 NL - 2280 HV Rijswijk
 Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,
 Fax: (+ 31-70) 340-3016

Authorized officer

SITCH, D

INTERNATIONAL SEARCH REPORT

Initial Application No

PCT/GB 96/02770

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>ASAIO JOURNAL, vol. 40, no. 3, 1 July 1994, pages 584-589, XP000498246 RAJASUBRAMANIAN G ET AL: "FABRICATION OF RESORBABLE MICROPOROUS INTRAVASCULAR STENTS FOR GENE THERAPY APPLICATIONS" see page M584, abstract ---</p>	
A	<p>VACCINE, vol. 12, no. 1, 1 January 1994, pages 5-11, XP000578212 MORRIS W ET AL: "POTENTIAL OF POLYMER MICROENCAPSULATION TECHNOLOGY FOR VACCINE INNOVATION" cited in the application see the whole document ---</p>	
A	<p>JOURNAL OF MICROENCAPSULATION, vol. 12, no. 1, 1 January 1995, pages 59-69, XP000486815 SAH H K ET AL: "BIODEGRADABLE MICROCAPSULES PREPARED BY A W/O/W TECHNIQUE: EFFECTS OF SHEAR FORCE TO MAKE A PRIMARY W/O EMULSION ON THEIR MORPHOLOGY AND PROTEIN RELEASE" see page 59, abstract ---</p>	
A	<p>PHARMACEUTICAL RESEARCH, vol. 8, 1991, page S151 XP002026578 NELLORE ET AL: "APPLICATION OF BIODEGRADABLE MICROSPHERES TO HEPATITIS B SURFACE ANTIGEN VACCINATION SYSTEM" see abstract PDD7098 -----</p>	

INTERNATIONAL SEARCH REPORT

International application No.

PCT/GB 96/02770

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 42-46
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 42 to 46 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

information on patent family members

nal Application No

PCT/GB 96/02770

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9524929 A	21-09-95	AU 2121495 A CA 2190121 A	03-10-95 21-09-95
WO 9520660 A	03-08-95	CA 2181832 A EP 0740704 A	03-08-95 06-11-96
WO 9423738 A	27-10-94	AU 6707194 A CA 2160878 A EP 0696200 A JP 8510639 T	08-11-94 27-10-94 14-02-96 12-11-96